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14. ABSTRACT

The thaxtomins are dipeptide phytotoxins produced by *Streptomyces acidoscabies* and related species. A novel feature of these compounds is the presence of a 4-nitrotryptophan moiety. The thaxtomin gene cluster contains two peptide synthetase genes (*txtA, txtB*), two cytochrome P450 genes, and a nitric oxide synthase (NOS) gene. The NOS gene is hypothesized to play a role in the formation of 4-nitrotryptophan. Both the mechanism and the timing of the nitration reaction are currently unknown. To determine the timing of the nitration reaction, we cloned the adenylation domains of *txtA* and *txtB*, and showed that the proteins can be overproduced in soluble form in *E. coli*. This should allow the substrate specificity of these adenylation proteins to be determined in future studies. We also used the NOS gene from the thaxtomin gene cluster to probe several species of *Streptomyces* that produce metabolites whose biosynthesis might involve a NOS. These investigations led to the cloning of a NOS gene from *S. alanosinicus*, which produces the antitumor agent L-alanosine. A transformation system was then developed for *S. alanosinicus* and the NOS gene was disrupted by single crossover insertion. The effect of NOS gene disruption on L-alanosine production is currently under investigation.

15. SUBJECT TERMS
Thaxtomin, Streptomyces, nitric oxide synthase, alanosine

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FINAL REPORT

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PRINCIPAL INVESTIGATOR: Ronald J. Parry

INSTITUTION: Rice University

GRANT TITLE: Investigations of Thaxtomin Biosynthesis

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OBJECTIVES: (1) To elucidate the timing of nitro group introduction during formation of the 4-nitrotryptophan moiety of the thaxtomins and (2) to investigate the potential involvement of nitric oxide synthases in the formation of other natural products.

APPROACH: The thaxtomins are cyclic, dipeptide phytotoxins produced by *Streptomyces acidoscabies* and related species. A novel feature of these compounds is the presence of a 4-nitrotryptophan residue. The thaxtomin gene cluster has been shown to contain two peptide synthetase genes, two cytochrome P450 genes, and a nitric oxide synthase (NOS) gene. The NOS gene is hypothesized to play a role in the formation of 4-nitrotryptophan. Both the mechanism and the timing of the nitration reaction are currently unknown. In order to elucidate the timing of 4-nitrotryptophan formation, we propose to determine the substrate specificity of the peptide synthetases TxtA and TxtB found within the thaxtomin biosynthetic gene cluster and thereby determine whether tryptophan is nitrated before or after incorporation into the diketopiperazine ring of the thaxtomins. In order to assess the involvement of nitric oxide synthases in the biosynthesis of other natural products, we propose to use the nitric oxide synthase (NOS) gene from the thaxtomin gene cluster to probe the genomes of several *Streptomyces* species that produce natural products whose structures suggest the possible involvement of a NOS in their formation.

ACCOMPLISHMENTS: The adenylation (A) domains of the two peptide synthetase genes *txtA* and *txtB* found in the thaxtomin gene cluster were amplified by PCR and cloned into the *E. coli* expression vector pFLAG-CTC. The two FLAG plasmid constructs containing the *txtA* and *txtB* A domains

were introduced into *E. coli* and experiments to overproduce the A domains were carried out. Both expression plasmids produced soluble protein. Once soluble proteins were obtained, we synthesized 4-nitrotryptophan from commercial 4-nitroindole by literature methods. Studies with the 4-nitrotryptophan and the overproduced TxtA and TxtB A domains are in progress.

To accomplish the second objective, the genomes of several promising *Streptomyces* strains were probed by Southern blotting with the thaxtomin NOS gene. These experiments led to the successful cloning of a NOS gene from the L-alanosine producer, *S. alanosinicus*. Attempts to detect a NOS gene in the remaining *Streptomyces* strains by either Southern blotting or by PCR failed. The potential role of the *S. alanosinicus* NOS gene in L-alanosine biosynthesis was investigated in two ways. The first way involved the development of a transformation system for *S. alanosinicus*. Using this system, the NOS gene was successfully disrupted by means of a single crossover insertion. The effect of this disruption on L-alanosine production is under investigation. In addition, a cosmid library of *S. alanosinicus* DNA was created and several cosmids containing the *S. alanosinicus* NOS gene were identified by Southern blotting. One of these cosmids was chosen for additional study and the DNA surrounding the NOS gene in this cosmid is currently being sequenced. This cosmid was also introduced into *S. lividans* to determine if the NOS-containing cosmid will confer L-alanosine production on this species.

CONCLUSIONS: Substantial progress has been made toward reaching both research objectives. The adenylations domains of TxtA and TxtB have been overproduced in soluble form in *E. coli* and purified by affinity chromatography. 4-Nitrotryptophan has been synthesized by literature methods and its ability to serve as a substrate for the A domains of TxtA and TxtB can now be assayed. The L-alanosine producer has been shown to contain a NOS gene and an *S. alanosinicus* strain with the NOS gene disrupted has been created. This strain will allow an assessment of the effect of NOS gene disruption on L-alanosine production

SIGNIFICANCE: An extension of these studies will provide important information on the timing of the nitration reaction associated with thaxtomin biosynthesis. This information should assist the elucidation of the mechanism of the nitration reaction. Prokaryotic genome sequencing has revealed the presence of NOS genes in a number of Gram-

positive bacteria, but their functions in these organisms are largely unknown. The discovery of a NOS gene in *S. alanosinicus* provides the opportunity to investigate the potential role of a NOS in the biosynthesis of an N-nitroso compound. The development of a transformation system for the L-alanosine-producing organism will allow genetic manipulations in this strain that should facilitate elucidation of the function of the NOS gene. Significant progress in this direction has been accomplished by creation of a NOS-minus mutant.

PUBLICATIONS AND ABSTRACTS:

None thus far.